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REGULAR ARTICLE

DIVERSITY, ANTIMICROBIAL ACTIVITIES AND ASSOCIATED MICROBIOTA OF SOIL PENICILLIA FROM VIRGIN FOREST FLOOR

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ABSTRACT

The diversity of *Penicillium* spp. and associate mycobiota from different virgin forest floor in the Brahmaputra Valley, Assam, India was analyzed. Soil samples were collected from six different undisturbed forest floors together with seasonally flooded forest and also from agricultural fields. Samples were taken from the litter and from three soil core i.e. 0-5, 10-15 and 30-35 cm in depth. The isolated fungal species were identified based on morphological and reproductive characteristics. About 18 common fungal species from different soil samples were isolated dominated by *Penicillium* sp. and *Aspergillus* sp. The total fungal population found in the studied sites was 98.87 (±10.7) x10^3 CFU/g dry soils in all the seasons in top soils. The total CFUs of *Penicillium* were also highest among the species in all the sites (mean 18.73 ±1.1 x10^3 CFU/g; n=7) where 27.2 x10^3 CFU/g in summer and 11.6 x10^3 CFU/g dry soil in winter. Relative density of *Penicillium* sp. was also higher among the associated fungi although relative density of *Aspergillus* (23.83) was higher than *Penicillium* (19.39). Among the 30 isolates of *Penicillium,* few species have shown antimicrobial activity against the tested bacterial pathogens. The cultural filtrate of four different isolates showed antimicrobial activity against *Streptococcus bombycis*, *Aeromonas salmonicida, Staphylococcus aureus* and *E. coli* having inhibition zone of about ≥10mm. All the tested bacterial species were sensitive to six different *Penicillium* spp.

Keywords: Antimicrobial activity; diversity; mycobiota; *Penicillium* spp.

INTRODUCTION

Fungi play a major role in soil ecosystems and are the principal decomposers of forest litter or dung, fruits or other organic materials (Carlile et al. 2001). Majority of the soil fungi are well known as saprobes, decomposing organic matter and contributing to nutrient cycling, while several species form mycorrhizal associations and also act as harmful agents as plant pathogens (Martins et al. 2007). Several fungal species produces bioactive compounds, secondary metabolites and chemical models having pharmaceutical importance (Suay et al. 2000; Zhang et al. 2009). There are 23,000 known secondary metabolites, 42% of which are produced by *Actinobacteria,* 42% by fungi (*Penicillium* spp.) and 16% by other bacteria (Kutzner, 1986). The *Penicillium* spp. is among the most commonly occurring and economically important members of them. However, documentation of microbial diversity of the virgin places of Indo Burma Biodiversity hot spot has yet to be started. North east India is one of the centres of mega biodiversity region and possesses a vast potential of undiscovered organisms including *Penicillium* spp. (Myers et al. 2000).

Penicillium is an ascomycetous fungal genus with widespread occurrence in most terrestrial environments. About two hundred species are well described and most of them are soil inhabitants, food borne contaminants or food ingredients used in the preparation of cheese and sausages (Pitt et al. 2000; Houbraken et al. 2010). Many isolates produce diversified active secondary metabolites, including antibacterial (Rancic et al. 2006; Larsen and Knochel, 1997), antifungal substances (Jayashree and Sivagurunathan, 1999), immuno-suppressants and also potent mycotoxins (Frisvad et al. 2004).

Thousands of *Penicillium* isolates have probably been screened in bioprospecting programs, and new bioactive metabolites continue to be discovered (Larsen and Knochel, 1997; Maskey et al. 2003), indicating their current importance as sources of high amounts of novel bioactive molecules to be used by pharmaceutical industry.

In the present study, we have isolated a number of *Penicillium* spp. from various locations together with its associated mycobiota from virgin soils and also screened for novel fungal natural products targeting at metabolites with biotechnological applications for the pharmaceutical industry (Bordoloi et al. 2001). The soils studied here derived from different locations in upper Brahmaputra Valley of Assam, India. The aims of the presented work were (i) to identify the most prominent members of the fungal communities in forest lands and (ii) to screen the antimicrobial activity of the *Penicillium* isolates collected from the soil samples.

MATERIAL AND METHODS

The studied sites

All the sample collected sites of Assam viz. Burhi Dihing (S1), Silapothar (S2), Majuli (S3), Giban Wildlife Sanctuary (S4), Bura Pahar (S5), Titabor (S6), Bogibeel (S7) were geographically different with foothills, to marshy lands, dry lands, urban and flood affected area. The present studied sites represent the middle and upper part of the state. The state experiences a very hot - humid weather during summer with an average temperature of 30°C (max. 38.5°C. min. 7°C). The annual rainfall ranges between 1500 mm to 2600 mm with moderate humidity (75%). A large part of the districts is covered by forest but falling a constant danger of denudation and deforestation due to the large felling of trees for timber, firewood, annual flood. The physico-chemical characteristics of soil differ from place to place. These forests receive abundant rainfall and support a vast variety of floral and faunal biodiversity. Due to the diverse climatic and topographic conditions, Assam forests support a vast floral diversity.

Collection and isolation of Penicillia and associated mycobiota

Soils from different depths of various sites (S1-S7, table 1) were collected for isolation of the species of Penicillia and associated myco-biota. The soil samples were collected in the polythene bags from the different depth (eg. top soil, 10cm, 20cm, 30cm and 50cm). The species were also collected from preserved and degraded fruits (S8-S10). Dilution plate technique was adopted for the isolation of fungal strains from the soil samples (Waksman, 1922). For the diversity analysis and total fungal population, the soil samples of different depths were mixed thoroughly and used for the stock solution. The samples were then mixed with sterile distilled water and a series of dilutions were made. From the dilutions (10^{-3}), 0.5ml samples were pipetted onto Potato Dextrose Agar in Petri plates and incubated at 26°C for three days. Isolation of the strains was carried out using the following standard methods given in the Manual of Microbiological Methods (Buchanan and Gibbon, 1974; Pitt, 1979) and Microbiological Methods (Collins and Lyne, 1989). Specific culture media (Potato Dextrose Agar, Malt Extract Agar etc) were also used for isolation of species Penicillia and associated mycobiota (Frisvad, 1993; Grammer, 1976). The isolated species were maintained in PDA media for further study. Preliminary identification of the genus was made on

morphological and reproductive characters with the help of standard manuals, books (Raper et al. 1949; Gilman, 1966; Subramanian, 1971, Ellis, 1976; Barnett and Hunter, 1972) and literature (Dorge et al. 2000). The isolated species were preserved and an isolate code number was given for further study.

Table 1 Soil samples collected sites

Collection sites	Samples	Code No
Burhi Dihing, Tinsukia	Soil	S1
Silapothar, Dhemaji	Soil	S2
Majuli, Jorhat	Soil	S3
Giban Wildlife Sanctuary, Jorhat	Soil	S4
Bura Pahar, Golaghat	Soil	S5
Titabor, Jorhat	Soil	S6
Bogibeel, Dibrugarh	Soil	S7

Ecological characteristics

From the different sample collection sites and also in different depth, each Penicillia isolates and the associated mycobiota were identified up to genus and noted for further analysis. The ecological characteristics of the isolated *Penicillium* spp. and other associated mycobiota were analyzed to get the quantitative ecological data like frequency, density, abundance and relative density by the standard ecological methods (Christensen, 1981; Sarma, 2001).

Antimicrobial activity

Among the isolates of *Penicillium,* few species having coloured secondary pigments/metabolites on the reverse side of the colony and also non pigmented colonies were taken for antimicrobial screening against certain clinical bacterial pathogens (Zhelifonova et al., 2010). The crude extracts of *Penicillium* were investigated for antimicrobial activity against bacterial pathogens i.e. *Streptococus bombycis, Aromonas salmonicida, Staphylococcus aureus* and *Echerichia coli* using the agar disc diffusion method with Petri dish template system inoculated with the assayed microorganisms, followed by incubation at 32^0C for 24 h (Suay et al., 2000). *Penicillium* methanolic extracts were prepared by mixing 2

ml of the production culture (Potato Dextrose Broth, PDB) with 2 ml of 100% methanol, shaking for 15 min and then centrifuged at 1500 g for 15 min. Two-ml aliquots of the methanol extracts were evaporated to half their volume in order to increase the concentration of the metabolites and reduce any toxic effect due to the solvent (Suay *et al,* 2000). A sterilized filter paper disc (1cm diameter) were dipped into the solution and placed on the media surface (PDA). The bacterial cultures grown in nutrient broth were inoculated (0.5ml) on the surface of the media and gently spread around the disc through the help of glass spreader. Sterilized distilled water was used as negative control instead of stock solution for the antimicrobial activity. However, streptomycin powder (0.05% w/v) was also used for standard positive control in the test (Silva et al. 2004).

Data analysis

All the data shown in the table and figure were calculated with the help of Microsoft Excel programme and statistical data analysis software Prism and Origin Pro 8.0 version. Graphs and bar diagrams were made with the help of the statistical software Origin.

RESULTS AND DISCUSSION

Diversity of *Penicillium* and associated fungi

From the ten different collection sites/materials, a total of 30 different isolates of *Penicillium* has been isolated. Twenty two species of different soil fungi including *Penicillium* were isolated from the soil samples collected from the different sites. The common associated mycobiota of *Penicillium* and their availability in different sites are shown in table 2. The genus *Penicillium* and *Aspergillus* were dominant in all soil samples although their relative densities were different in different seasons. *Aspergillus* sp., *Mucor* sp., *Cladosporium* sp., *Cunninghamella* sp., *Curvularia* sp., *Drechslera* sp., *Fusarium* sp., *Gliocladium* sp., *Alternaria* sp., *Humicola* sp., *Paecilomyces* sp. etc were the dominant associated mycoflora of *Penicillium* in all the samples. These fungal genera were identified according to their vegetative and reproductive characters following standard manuals and references. The total CFU of all the fungal genera in the different sites are shown in the table 3.

Some other least dominated mycoflora were *Periconia* sp., *Pestalotiopsis* sp., *Rhizoctonia* sp., *Scelerotium* sp., *Sepedonium* sp., *Trichoderma* sp., *Verticillium* sp. The frequency of *Penicillium sp.* and *Aspergillus sp*. were the maximum (100%) in all the studied populations. The relative density of *Penicillium* was higher in all the sites except in summer seasons where the relative density of *Aspergillus* was more than *Penicillium* (Table-4). However, the abundance of the fungal genera in summer/rainy was significantly higher in the sites (Fig. 1). The frequency of *Pestalotiopsis* sp., *Cladosporium* sp., *Drechslera* sp., *Scelerotium* sp., *Rhizoctonia* sp. and *Periconia* sp. was very low in the collection sites.

Table 2 Soil mycobiota of sample collected sites

Sl No	Fungal genera	Studied sites (+, Present; -, absent)						
		S1	S2	S3	S4	S5	S6	S7
1	*Alternaria* sp.	+	+	+	+	+	+	+
2	*Aspergillus* sp.	+	+	+	+	+	+	+
3	*Chaetomium* sp.	+	+	+	-	+	+	+
4	*Cladosporium* sp.	+	+	+	+	+	-	-
5	*Cunninghamella* sp.	+	+	+	+	+	+	+
6	*Curvularia* sp.	+	+	+	+	+	+	+
7	*Drechslera* sp.	+	+	+	-	+	+	+
8	*Fusarium* sp.	+	+	+	+	+	+	+
9	*Gliocladium* sp.	+	+	+	+	+	+	+
10	*Humicola* sp.	+	+	+	+	+	+	+
11	*Mucor* sp.	+	+	+	+	+	+	+
12	*Paecilomyces* sp.	+	+	+	+	+	+	+
13	*Penicillium* sp.	+	+	+	+	+	+	+
14	*Periconia* sp.	+	+	+	+	-	+	-
15	*Pestalotiopsis* sp.	+	+	+	+	+	+	+
16	*Rhizoctonia* sp.	+	+	+	+	+	+	+
17	*Rhizopus* sp.	+	-	+	+	+	+	+
18	*Scelerotium* sp.	+	+	+	+	+	-	-
19	*Sepedonium* sp.	+	+	+	+	+	+	+
20	*Trichoderma* sp.	+	+	+	+	+	+	+
21	*Verticillium* sp.	+	+	+	+	+	+	+
22	*Mycelia sterilia*	+	+	+	+	+	+	+

Table 3 Fungal population in the different sites in all the four seasons (CFUx10^3/g soil).

Fungal genus	Sites						
	S1	S2	S3	S4	S5	S6	S7
Penicillium sp.	18.9±3.6	19.4±5.8	19.4±3.8	17.4±7.5	20.3±1.6	17.3±2.3	18.45±1.9
Aspergillus sp.	17.4±6.6	19.6±8.2	19.1±7.0	21.6±7.9	20.4±8.0	11.25±2.0	15.95±6.0
Fusarium sp.	8.4±3.2	8.2±4.0	9.8±1.3	6.9±2.5	7.6±2.5	6.2±2.9	9.25±2.5
Curvularia sp.	2.9±0.6	2.8±0.4	4.7±1.5	5.1±4.7	5.1±1.6	2.5±1.3	4.45±4.0
Alternaria sp.	5.7±2.9	5.65±3.6	6.05±2.9	4.05±2.0	6.65±1.5	5.75±2.2	6.55±1.9
Trichoderma sp.	9.6±2.0	9.1±1.3	8.85±1.1	6.65±1.3	7.35±1.0	6.1±0.9	6.75±2.0
Cunninghamella sp.	1±0.4	1.3±0.8	1.5±0.3	3.9±4.4	2.3±1.0	1.3±0.6	2.05±1.7
Verticillium sp.	2.1±0.9	1.8±1.0	1.7±0.9	2.45±1.7	0.9±0.4	1.75±1.1	1.65±1.5
Gliocladium sp.	1.7±0.4	1.1±0.6	1.15±1.5	2.45±1.5	2.05±0.7	0.8±0.1	2.15±1.6
Humicola sp.	2.8±0.5	2.7±0.8	3±1.1	2.6±1.7	2.6±1.8	2.7±1.4	1.75±2.0
Paecilomyces sp.	3.6±1.0	2.5±1.0	2.65±0.9	1.8±1.0	2.55±1.7	1.45±0.7	1.6±0.6
Pestalotiopsis sp.	0.9±0.6	1.2±0.1	1.25±1.0	2.3±3.5	0.85±0.4	0.9±0.7	0.95±0.3
Sepedonium sp.	1.2±1.2	1.05±0.5	1.3±0.9	0.75±0.4	2.45±3.0	1.6±0.5	1.15±0.1
Cladosporium sp.	3.7±2.4	2.55±1.7	2.3±0.9	1±0.5	2.55±1.9	0.15±0.3	0±0
Drechslera sp.	0.8±0.3	0.6±0.1	1.1±0.9	0±0	1.05±0.5	0.6±0.3	0.95±1.0
Scelerotium sp.	2.1±1.3	1±0.4	1.15±1.2	1.1±0.4	1.1±1.0	0±0	0.25±0.5
Rhizoctonia sp.	3.6±1.5	3.05±1.7	2.1±1.2	3.75±1.2	3.95±2.5	2.4±1.4	1.85±0.6
Periconia sp.	1.1±1.1	1.55±0.8	1.8±1.0	1±0.8	0.8±1.0	1±0.8	0.25±0.5
Mucor sp.	6.2±2.7	6.3±2.7	6.65±1.4	6.05±1.8	5.95±3.4	5.15±3.5	6.2±3.3
Chaetomium sp.	1.7±0.3	2.15±1.1	2.6±0.7	0±0	1.5±0.5	1.55±1.3	1.25±0.7
Rhizopus sp.	3.9±2.3	0.3±0.6	3.5±1.9	2.05±1.5	3.2±1.6	3.3±1.5	3.65±2.5
Mycelia sterilia	6.1±1.4	6.45±2.9	6.8±2.7	4.8±1.3	7.35±2.2	4.95±1.3	5.75±1.4
Total	106.05	100.25	108.35	97.4	108.5	78.7	92.85

Table 4 Abundance and relative density of *Penicillium* and its associated mycoflora in four different seasons

Fungal species	Abundance (%)				Relative Density (%)			
	Pre-monsoon	Rainy	Autumn	Winter	Pre-monsoon	Rainy	Autumn	Winter
Penicillium sp.	18.6	27.6	11.6	11.6	23.78	20.6	11.9	14.4
Aspergillus sp.	21.2	31.6	20.4	12.2	27.10	23.61	20.98	15.17
Fusarium sp.	10	4.75	6.8	7	12.78	2.84	6.99	8.70
Curvularia sp.	0.27	11.6	5.8	2.25	1.53	8.66	5.96	2.23
Alternaria sp.	1.2	5.6	5.4	5	1.53	4.18	5.55	4.97
Trichoderma sp.	13.5	6.8	6	8.4	6.90	5.08	6.17	10.44
Cunninghamella sp.	2	10.4	3.4	1.5	1.53	7.77	3.49	0.74
Verticillium sp.	1.66	5	2.25	3.33	1.27	3.73	1.85	2.48
Gliocladium sp.	5	4.6	2.2	1.25	2.55	3.43	2.26	1.24
Humicola sp.	1.66	1.5	4.4	3.8	1.27	0.89	4.52	4.72
Paecilomyces sp.	2	4	5	1.33	1.53	2.39	2.05	0.99
Pestalotiopsis sp.	2	1.5	1.5	7.6	0.51	0.44	0.61	9.45
Sepedonium sp.	1	1.5	3	1.66	0.25	0.44	1.23	1.24
Cladosporium sp.	2	2.25	1.5	1.33	1.02	1.34	0.61	0.99
Drechslera sp.	0	0	0	0	0	0	0	0
Scelerotium sp.	2	1.66	3	1.5	2.04	0.74	1.23	0.74
Rhizoctonia sp.	2.5	4	4.8	4.2	2.55	2.98	4.93	5.22
Periconia sp.	2.66	1	3	1	2.04	0.14	1.85	0.49
Mucor sp.	3.4	7.2	6.4	7.2	4.34	5.38	6.58	8.95
Chaetomium sp.	0	0	0	0	0	0	0	0
Rhizopus sp.	2	2.25	4.2	3	0.51	1.34	4.32	2.23
Mycelia sterilia	3.8	5.2	6.6	4.5	4.85	3.88	6.79	4.47

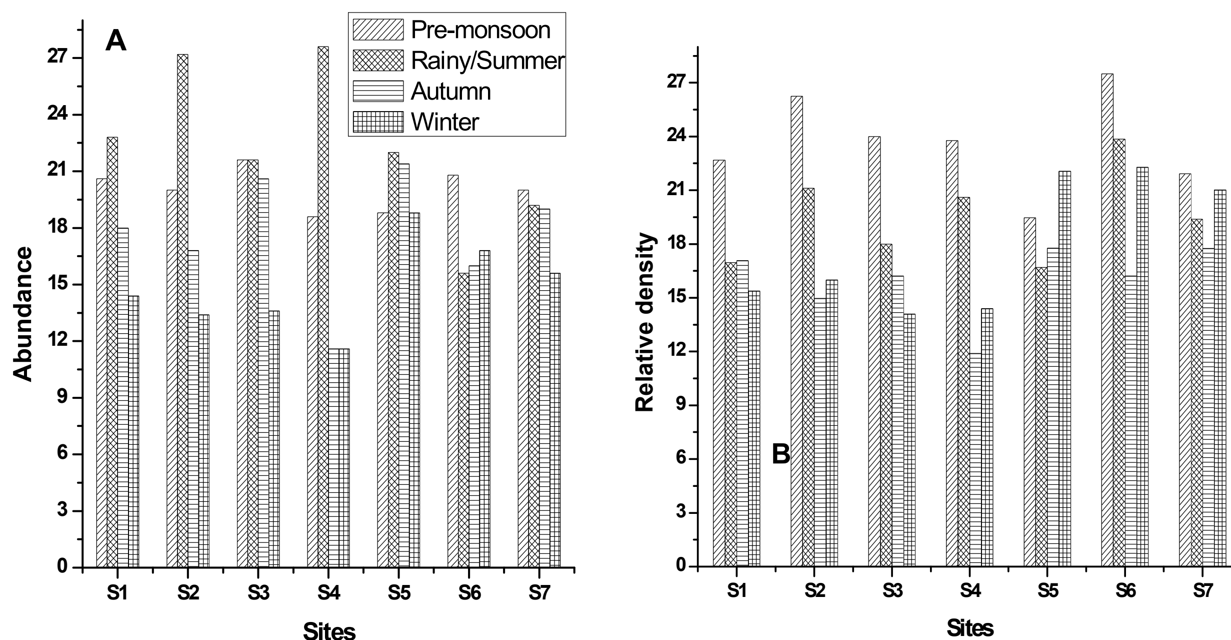

Figure 1 Abundance (A) and relative density (B) of *Penicillium s*p. in different seasons

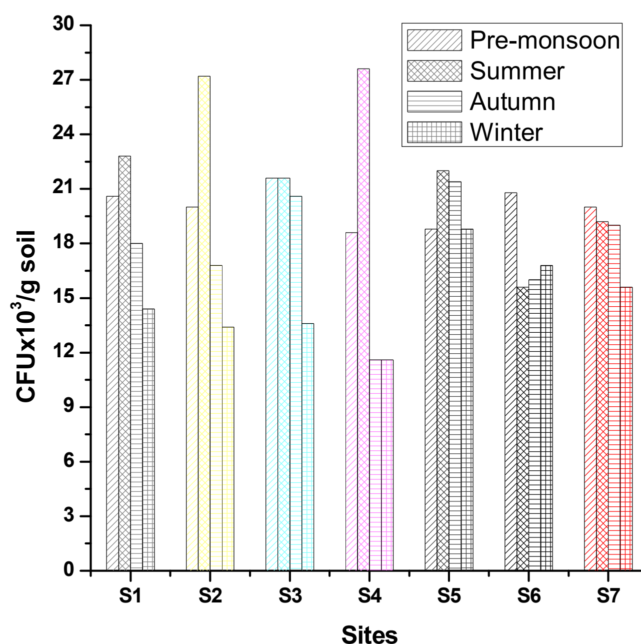

Figure 2 Population of *Penicillium* sp. in different seasons (S1-S7)

Distribution of *Penicillium* spp.

The total fungal population found in the studied sites (S1-S7) was 98.87 (±10.7) x10^3 CFU/g dry soils in all the seasons. The total CFU of *Penicillium* were also highest among the species in all the sites 18.73 (±1.101784) x10^3 CFU/g dry soil (n=7) where 27.2 x10^3 CFU/g

in summer and 11.6 x10^3 CFU/g dry soil in winter followed by *Aspergillus* and sterile mycelia in the studied sites (Fig. 2). Relative density of *Penicillium* sp. was also higher among the associated fungi although relative density of *Aspergillus* (23.83) was higher than *Penicillium* (19.39) in summer seasons in S7.

Maximum numbers of *Penicillium* sp. were found in top soils (0-9cm depth) in all the sample collected sites. Highest number of CFU of *Penicillium* sp. was recorded in S2 in summer seasons in top soil followed by autumn, pre monsoon and winter respectively (Fig. 3). Among the sample collected sites, highest population of *Penicillium* (31x10^3 CFU/g soil) was found in the site S4 in summer.

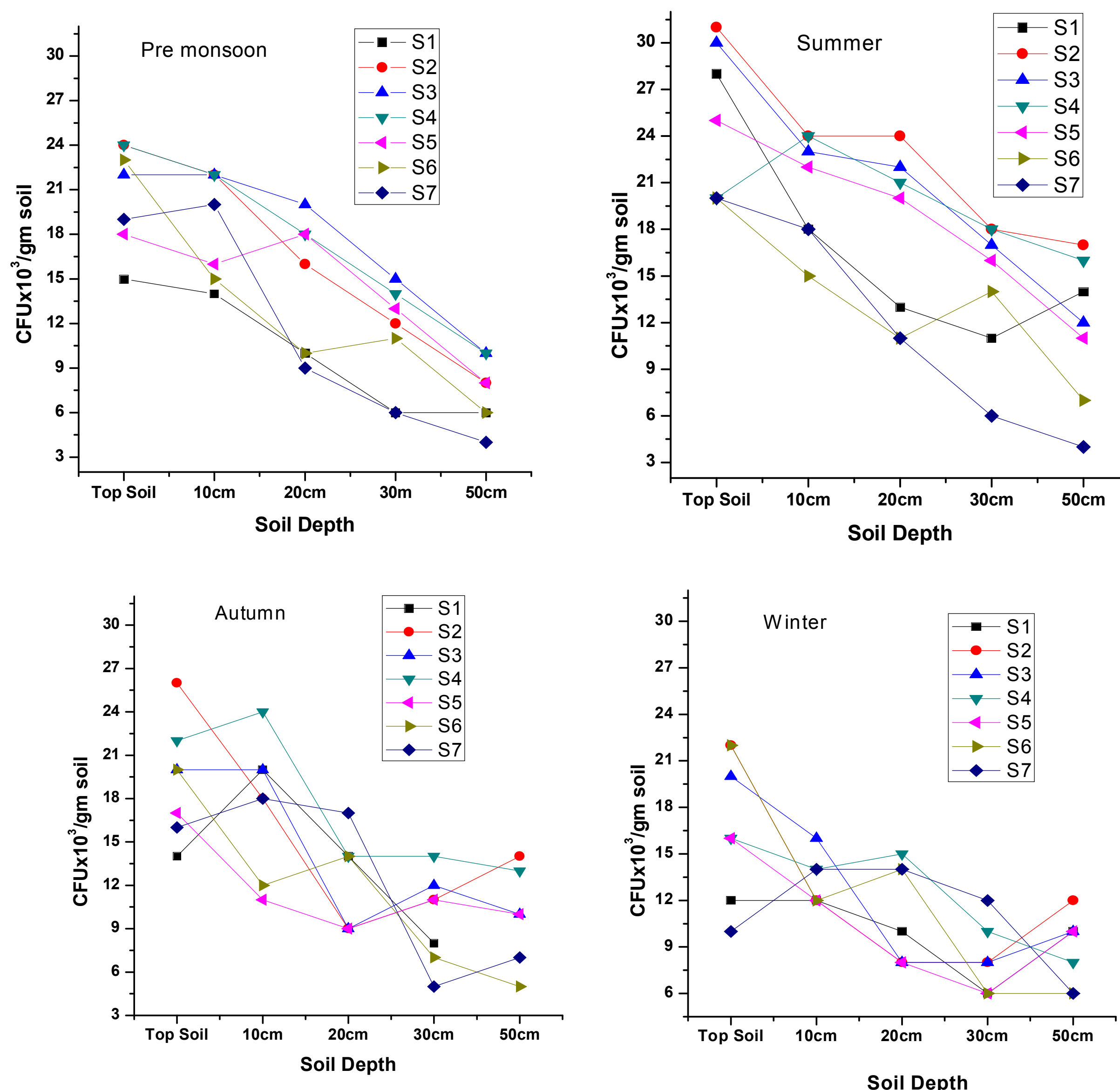

Figure 3 Distribution of *Penicillium* sp. in different soil depth (0-50cm) in different sample collected sites

Antimicrobial activity

Among the 30 isolates of *Penicillium,* few species have shown antimicrobial activity against the tested bacterial pathogens. The isolates P3, P5, P6, P10, P14, P19, P23 and P25 had shown antimicrobial activity against different tested bacterial pathogens. All the bacterial species were sensitive to P23 and P6 although their ranges of activity were different. *S. bombycis* and *E. coli* was more sensitive to P23 cultural filtrate with an inhibition zone of more than 10mm in diameters followed by P6 cultural filtrates (≥7mm). *S. bombycis* was sensitive to P23, P25 (≥10 mm), P6 (≥7mm), P14 and P3 (≥4mm) isolates. *A. salmonicida* was only sensitive to the P6, P23 and P10 isolates.

Table 5 Antimicrobial activity of some *Penicillium* isolates against pathogenic bacteria

Penicillium isolates	Inhibition zone (mm)			
	Staphylococcus aureus	*Echerichia coli*	*Streptococus bombycis*	*Aromonas salmonicida*
P3	++	++	++	-
P5	++	-	-	-
P6	+++	+++	+++	++
P10	-	-	-	+
P14	+	-	++	-
P19	-	-	+	-
P23	++	++++	++++	++
P25	-	++	++++	-

+, 1-3mm; ++, 4-6mm; +++, 7-9; ++++, ≥10

Soil fungal diversity depends on a large number of factors of the soil such as pH, organic contents, and moisture (Alexander, 1977, Rangaswami and Bagyaraj, 1998). Diversity was found to be higher in the undisturbed land in summer seasons. Among the various genera of soil fungi of different soil collected sites *Penicillium* and *Aspergillus* was the most common genera that was distributed in all the types, indicating that it adapts easily to different environment as well (Wahegaonkar et al. 2011**)**.

Several isolated species viz. *Fusarium, Curvularia, Alternaria etc.* were involved in strong fungal associations and have dominant adaptative features as primary colonizers probably due to their capacity for the rapid invasion of the available substrate (Frankland,

1981). However, seasonality is one factor that was believed to affect the fungal community structure (Seephueak et al. 2010). Diversity of fungi communities in forest floor varied season wise although it is still unclear how the seasons affect fungal communities (Kennedy et al., 2006). As the occurrence of fungal species was regulated primarily by season which may be cause and effect operates via humidity and temperature (Nikolcheva and Bârlocher, 2005).

The samples collected in the summer season tended to be richer in species diversity and have a higher Shannon diversity index than samples collected in the dry season. On the contrary, Kodsueb et al. (2007) reported that the diversity of saprobic fungi on litter samples collected in the dry season had greater species richness than samples collected in the wet season, which suggest a humidity factor. Rayner and Todd (1979) also reported a greater variety and number of fungi during the dry season. High humidity was needed for the germination and dispersal of fungi (Pinnoi et al. 2006) and hence diverse population of fungi were reported in the summer. In this study we have shown that fungal communities during the wet season are more diverse. Thus, many factors affect the changes in community structure; for instance, the microclimate of the growing area, biological interaction within leaf litter, or substrate, microhabitat preference and host preferences (Lodge, 1997).

CONCLUSION

The results obtained from the study clearly indicated that maximum number soil *Penicillium* sp. were found in the soils during summer seasons rather than dry or winter season. The associated mycobiota of *Penicillium* had also higher population in rainy seasons. *Penicillium, Aspergillus* and *Fusarium* species were the most dominant fungal genera in all the season forming a group in the virgin terrestrial habit in the studied areas. The *Penicillium* species isolated from the virgin soils had also potential antimicrobial producing activity against clinical bacterial pathogens which may further be utilized for production of novel fungal compounds.

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